

Title: Remineralization and acid resistance of enamel lesions after
chewing gum containing fluoride extracted from green tea

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Short title: Effect of fluoride chewing gum on enamel

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Abstract: Objective: The aim of this study was to evaluate enamel remineralization and the acquisition of acid resistance by using sugar-free chewing gum containing fluoride extracted from green tea. **Methods:** Forty-five volunteers participated in a crossover, double-blind study and wore intra-oral appliances with human demineralized enamel. Subjects chewed fluoride chewing gum (FCG: 50µg fluoride) or placebo gum. Remineralization and acid resistance were evaluated using the mineral change value (ΔZ , in vol%·µm). Fluoride concentrations in saliva and remineralized enamel were analyzed. **Results:** The peak salivary fluoride concentration was 3.93 ± 1.28 ppm (mean \pm SD). The elevated salivary fluoride concentration resulted in a higher fluoride concentration of 656 ± 95 ppm in the remineralized region versus 159 ± 26 ppm for placebo gum ($P < 0.001$). After remineralization, the ΔZ of the FCG group was higher than that

of the placebo gum group. After an acid challenge, ΔZ of the FCG group was lower than the placebo gum group. Both ΔZ were statistically significant. **Conclusion:** FCG produced a superior level of remineralization and acid resistance, as compared to the placebo gum. The in situ results suggest that the regular use of FCG is useful for preventing dental caries.

Key words: Green tea extract, fluoride chewing gum, enamel, remineralization, acid resistance, sugar-free gum, dental caries

The use of fluorides has proven to be clinically effective in controlling dental caries in a large number of clinical trials, literature reviews and, more recently, meta-analyses of studies involving the use of rinses, gels, varnishes, and dentifrices¹. The continual presence and availability of low levels of fluoride ions in the oral environment, especially at the saliva/enamel interface, helps to prevent dental caries and promote the remineralization of enamel that has been demineralized by acids produced by cariogenic bacteria^{2,3}. Methods that maintain a constantly elevated intra-oral fluoride concentration should have a preventive effect on dental caries. For this purpose, fluoride-containing chewing gum (FCG) and slow fluoride-releasing devices can be used as delivery methods. Unfortunately, no commercial slow fluoride-releasing device is available in Japan, and while FCG has been marketed in European Union countries, it is not available in

Japan⁴. The active fluoride component of FCG is usually NaF.

In Japan, the Pharmaceutical Affairs Act prohibits chemical fluoride materials as food additives. However, plant-derived fluoride for eating or drinking on a daily basis can be used as food additives. Plants in the family *Theaceae*, such as *Camellia sinensis*, the so-called tea tree, contain high concentrations of fluoride in their leaves and infusions⁵.

Traditionally, *in vitro* or animal studies of the relationship between dental caries and tea extracts have focused on the antibacterial activity of polyphenolic compounds⁶⁻⁹. Recently, *in situ* studies have begun to explore the effects of tea extracts as a promising preventive measure to reduce dentin erosion and abrasion¹⁰. However, little is known about the effects of tea extracts on the remineralization of enamel and the acquisition of acid resistance. Camellia extract MJ (Taiyo Kagaku) is a green tea extract that has

high fluoride content and can be used as a food additive in Japan. It contains over 1500 ppm of fluoride. It was previously reported that a cup of green tea contains 0.5-1 g of catechins/L ¹¹. In contrast, Camellia extract MJ contains approximately 0.06 g of catechins/L.

Using Camellia extract MJ as the source of fluoride in chewing gum, this paper investigated its effects on demineralized enamel to improve dental health. We evaluated enamel remineralization and the acquisition of acid resistance using sugar-free chewing gum containing the green tea extract.

Subjects and Methods

Study design and subjects

This study was a double-blind, randomized, crossover design. It was approved by the Dental Research Ethics Committee of Nagasaki University (authorized number: 0735) and the Koseikai Yostubashi Clinic Human

Research Ethics Committee (authorized number: 20070326-4-01), and informed consent was obtained from each subject, and was conducted under the principles of the Declaration of Helsinki. This study recruited 45 healthy adults (20 males, 25 females; age 23–55 years) from university students (10 males, 11 females; age 23–35 years) and the general public (10 males, 14 females; age 32–55 years). None of the subjects were using medications that might have affected the salivary flow rate.

Preparation of intra-oral appliances and enamel subsurface lesions

Removable mandibular appliances were prepared for each subject. From human sound first premolar teeth extracted for orthodontic purposes, two enamel blocks (ca. $3 \times 4 \times 2$ mm) were cut and covered with acid-resistant nail varnish, except for the enamel surfaces. Experimental subsurface lesions were created using demineralizing buffer containing 0.1 M lactic

acid, 1% carboxymethyl cellulose, 3.0 mM calcium, 1.8 mM phosphate, pH 4.5 for 72 hours at 37°C (20 ml/specimen) ¹². After demineralization, one-third of each enamel specimen was covered with nail varnish as the demineralized (DEM) area and the specimen was mounted in an appliance with dental sticky wax (Fig. 1).

In situ experimental protocol

The experimental procedure is outlined in Fig. 2. Pairs of enamel specimens derived from the same tooth were used for each trial period.

The sugar-free chewing gums were provided by Meiji Seika Kaisha. The gums were pellet type with a maltitol coating and weighed 1.4 ± 0.1 g/piece.

One of the gums contained 1.17% (w/w) green tea extract “Camellia Extract MJ” (25 µg fluoride /piece), but was identical to the placebo gum in all other respects. The compositions of the sugar-free gums are shown in

Table 1. The subjects were instructed to chew two pieces of the sugar-free gum at the same time for 20 minutes, twice per day for 4 weeks. The intra-oral appliances were put in the mouth just before chewing and kept in the oral environment for another 20 minutes after chewing. In total, the appliances were kept in the oral environment for 80 minutes per day, and when the appliances were removed, they were stored in a moist plastic container at room temperature. The subjects were instructed not to eat, drink, smoke, or perform oral hygiene procedures while wearing the appliances and were prohibited from using dentifrices or mouthwashes containing fluoride throughout the study period. Each treatment period lasted 4 weeks with a 1-week washout period between treatments.

Acid challenge test (ACT)

After the intra-oral treatment phase, the enamel specimens were removed

from the appliances. Another one-third of each enamel surface was covered with nail varnish as the remineralized (REM) area and the remaining one-third of the surface was treated with the demineralizing buffer again as the ACT area for 72 hours at 37°C.

Microradiography

After the ACT, the enamel specimens were cut into approximately 200- μm sections with a water-cooled diamond microtome for hard tissues (Series 1000 Deluxe, USA). Then, microradiography were performed, as described previously^{13, 14} using an x-ray radiation system (HB-50, Hitex) at 2 kV, 20 mA, for 20 minutes. Each radiographic image of a lesion was scanned three times at different locations with a density meter (PDM-5, Konica Minolta). The lesion parameters such as mineral loss values (ΔZ in vol%· μm) and lesion depth (μm) obtained from these three scans were

averaged and the mineral loss values were used as the main parameter for the specimens. ΔZ of each area of specimen (DEM, REM and ACT) were represented in ΔZ_{dem} , ΔZ_{rem} , and ΔZ_{act} , respectively. The mineral change values during the *in situ* remineralization ($\Delta Z_{REM} = \Delta Z_{dem} - \Delta Z_{rem}$) and the ACT ($\Delta Z_{ACT} = \Delta Z_{act} - \Delta Z_{rem}$) were analyzed.

Saliva collection and measuring the salivary fluoride concentrations

Whole mixed saliva samples for fluoride measurements were collected before (unstimulated saliva) and 0.5, 1, 3, 5, 10, 15, and 20 minutes after chewing two pieces of FCG. Saliva collection was performed one week ahead of the test period. The subjects were prohibited from eating, drinking, and using dentifrices or mouthwashes containing fluoride beginning one hour before the start of collection. In each case, twice the volume of total ion strength adjustment buffer (TISAB) IV ¹⁵ was added to

the collected saliva sample before measurement. Deposit-free supernatants were analyzed using a fluoride ion-specific electrode (F-53/6561-10C, Horiba, Japan). The mean amount of fluoride released from the FCG group (μg) was calculated from the salivary fluoride concentration and the volume of the saliva samples.

Measuring the fluoride content of remineralized enamel

From each treatment group, ten enamel specimens with sufficient remaining REM area for analysis were selected. The specimens were dissolved in 1 ml of 0.5 M HClO_4 for 2 minutes for surface layer analysis and for an additional 6 minutes for the subsurface layer analysis. After each etching, 2 ml of TISAB IV were added and the fluoride ion concentration was measured using a fluoride ion-specific electrode in the same manner as described above. The calcium ion concentration of each

sample was measured with 0.5% LaCl_3 using an atomic absorption spectrometer (Z-5300, Hitachi, Japan). The following equations were used to calculate the acid etched depth and the fluoride content of each layer sampled from the enamel specimens. The calculations used a density of 2.95 g/cm^3 for enamel¹⁶ and a 37.5% calcium concentration of the enamel. Since the exact density of partially demineralized enamel is not known, the two parameters calculated are approximations.

$$\text{Acid etched depth } (\mu\text{m}) = \text{Mass of calcium } (\mu\text{g}) / [37.5\% \times 2.95 (\text{g/cm}^3) \times \text{surface area } (\text{mm}^2)] \times 10^3$$

$$\text{Fluoride content (ppm)} = [\text{Mass of fluoride } (\mu\text{g}) / \text{Mass of enamel in specimen } (\mu\text{g})] \times 10^6$$

Statistical analysis

The parameters ΔZ_{REM} and ΔZ_{ACT} were evaluated using analysis of

variance with Tukey' s honestly significant difference for multiple comparisons (SPSS for Windows ver.12.0J). Independent t-tests were used to compare the fluoride concentrations of enamel between the two treatments. Statistically significant differences were set at a probability level below 5%.

Results

All 45 subjects finished the test period without dropping out. During sample preparation, one enamel specimen was damaged mechanically and was excluded from the analysis set. Ultimately, there were 44 subjects in the FCG group (20 males, 24 females; mean age 35.3 years) and 45 in the placebo group (20 males, 25 females; mean age 35.1 years). The mineral change values during the in situ remineralization and the ACT were shown in Table 2. The increase of the ΔZ_{REM} was significantly greater for

the FCG ($P<0.05$). And FCG resulted in significant resistance to the acid challenge compared with the placebo gum ($P<0.05$).

The mean salivary fluoride concentration while chewing two pieces of FCG was plotted as a function of time (Fig. 3). Salivary fluoride concentration peaked at 3.93 ± 1.28 ppm for 0.5–1 minute and remained above 0.18 ± 0.20 ppm while chewing for 20 minutes. These fluoride concentrations were markedly higher than the baseline fluoride concentration in saliva (0.05 ± 0.03 ppm). The fluoride contents of the remineralized regions were shown in Table.3. Fluoride contents in the FCG group were markedly higher ($P<0.001$) than in the placebo group both in the surface layers (depth ca. 30 μm) and in the subsurface layers (depth ca. 30–120 μm). Fig. 4 shows typical mineral profiles (Fig. 4A, 4B) and microradiographs after the ACT (Fig.4C, 4D); there was a laminar

structure in the FCG group (arrow in Fig. 4D), but not in the placebo group (Fig. 4C). The laminar structure was seen in 29 of the 45 samples from the FCG group and in 17 of the 46 samples from the placebo group.

Discussion

The continuous use of two pieces of FCG (25µg fluoride/piece) for 20 minutes, twice per day for 4 weeks, maintained markedly higher salivary fluoride concentrations than the baseline in this study. The significant elevation in the salivary fluoride concentrations (Fig. 3) probably contributed to the significant promotion of remineralization of early enamel lesions (Table 2). Importantly, exposure to FCG produced significantly higher acid-resistance from the surface to a depth of ca. 120 µm, presumably due to the promotion of remineralization and fluoride

intake to the remineralized enamel. Since the use of dentifrices and mouthwashes containing fluoride was prohibited during this study, the deposition of acid-resistant mineral was attributed to the fluoride ions released by the FCG. Supporting this finding, the fluoride content of the remineralized enamel lesions treated with FCG was at least twice as high as in the controls, from the surface to a depth of 120 μm .

Our study is premised on the hypothesis that ongoing exposure to low levels of fluoride enables significant remineralization of early enamel lesions. This concept has already been suggested by Featherstone¹⁷ and ten Cate¹⁸, *i.e.*, the frequent application of low-level fluorides effectively inhibits demineralization and enhances remineralization. In our study, appliances were kept in the oral environment for 80 minutes/day and during this period the subjects were instructed not to eat or drink.

Consequently, the clearance rate of fluoride would be much slower. Other FCG studies have demonstrated that the salivary fluoride concentration remained elevated for over 1 hour after chewing 0.25 mg F¹⁹, or 0.5 mg F²⁰ and for up to 4 hours after chewing 0.25 mg F²¹. Considering the promotion of remineralization and formation of acid-resistant mineral we observed in early enamel lesions, a very low fluoride content, *i.e.*, 0.05 mg F in FCG in the oral environment is satisfactory. Our findings concurred with those of Lamb *et. al.*²², who found that when salivary fluoride levels were increased after using FCG, remineralization was promoted, confers significant resistance to acid challenge. They also found that the fluoride uptake was significantly higher for the FCG-treated enamel lesions, compared with both control and sorbitol-treated lesions, up to a depth of

70 μm . Moreover, the mean peak salivary fluoride concentration was 4.19 ppm after chewing FCG containing 0.1 mg F/stick. The peak fluoride level is similar to our results.

The main reason for the acquisition of acid-resistant minerals might be related to the effects of fluoride ions during remineralization. Our study examined the effects of using FCG on demineralized enamel. The peak fluoride concentration in saliva was ca. 4 ppm and levels over 0.1 ppm were maintained while chewing FCG for 20 minutes. In this study, the elution rate of fluoride from the FCG was calculated to be about 77% (data not shown). Therefore, a fluoride-rich acid-resistant mineral probably formed within the crystal spaces of the remineralized enamel samples. The reduction in lesion porosity caused by the fluoride-rich mineral increases the enamel resistance to subsequent acid challenge. As Fig.4

shows even after remineralization, the surface mineral vol% is not as high as that of sound enamel (87 vol%). This means that remineralized lesions still have microspaces in the surface region. During the second acid challenge, the acids bypass the acid-resistant minerals located in the microspaces near the surface of the remineralized lesions and dissolve the underlining intact enamel that is not affected by demineralization/remineralization, where the acid resistance is lower than in the remineralized enamel. We found that the fluoride concentration in the surface and subsurface regions was 2- to 4-times higher (Table 3), respectively, which supports the concept that fluoride-rich mineral coats the intra-prismatic spaces in remineralized lesions. These results agree with reports of higher acid resistance and

fluoride content in arrested enamel caries than in sound enamel²³⁻²⁵, and this is probably one reason why the laminar pattern underneath the remineralized lesions was more pronounced in the FCG group after the acid challenge (Fig. 4). This laminar pattern was present in some of the control group because the original enamel probably contained high fluoride levels. This laminar structure is believed to be evidence of resistance to the demineralization of remineralized enamel²⁶⁻²⁸. Green tea catechins are known to have many anticariogenic effects^{11,29}. In this study, the peak and bottom salivary concentrations of total catechins were estimated to 0.12 and 0.006 g/L, respectively. These concentrations seemed to be unrealistically low to exert anticariogenic effects in human mouth. So the promoting of remineralization and acquisition of acid resistant property of enamel were mainly due to fluoride ion released from

FCG and the effects of catechins were minor. The FCG results are most applicable on the tooth to promote post eruptive maturation at and shortly after eruption or on early enamel caries without cavitation to arrest and even regress through remineralization. From the result of increasing the saliva flow rate, FCG may also be applicable to the patients who suffer from dry mouth.

We designed the chewing gum to release over 0.1 ppm fluoride in saliva after 20 minutes based on the results of Tumba and Curzon³⁰, who used a glass slow-release device and concluded that both deciduous and permanent caries indexes were significantly reduced in schoolchildren over 2 years. On completion of their trial, there were highly significant differences in the salivary fluoride concentrations at both 1 year and at the end of the trial. They found that the mean salivary fluoride

concentration was 0.11 ppm in the test group. From a clinical perspective, our data support the finding that the protective effect on demineralization derived from fluoride reactivity in early carious lesions—i.e., acquired acid resistance, as popularized by Koulourides²³—is the main factor affecting the decay-preventive effects of topical fluoride. FCG derived from green tea extract would be useful for providing topical fluoride to prevent dental caries.

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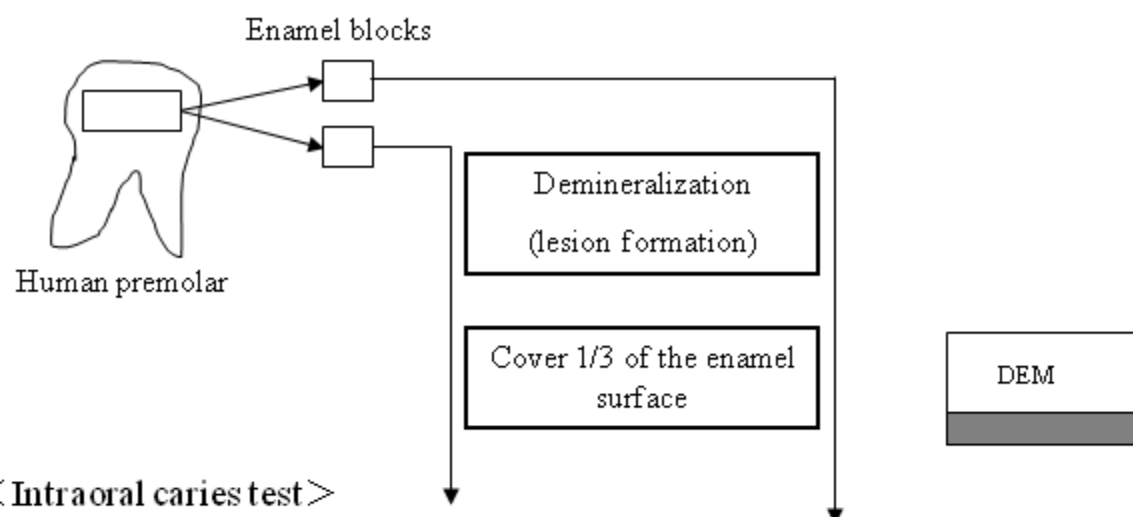
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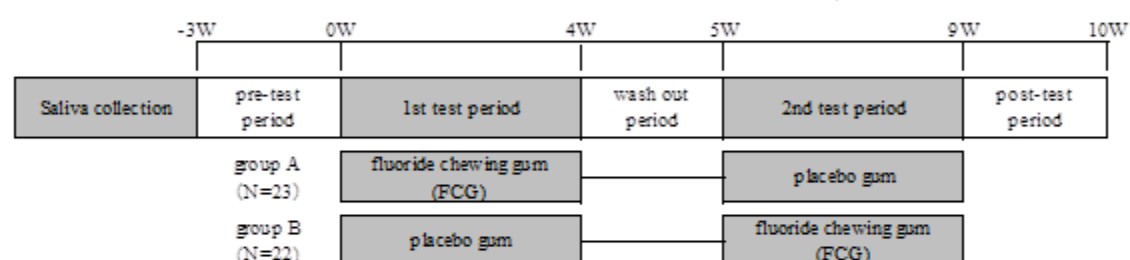


Figure 1: Intraoral photograph showing the intraoral appliance with a subsurface lesion in the enamel

< Preparation >



< Intra oral caries test >



< Analysis >

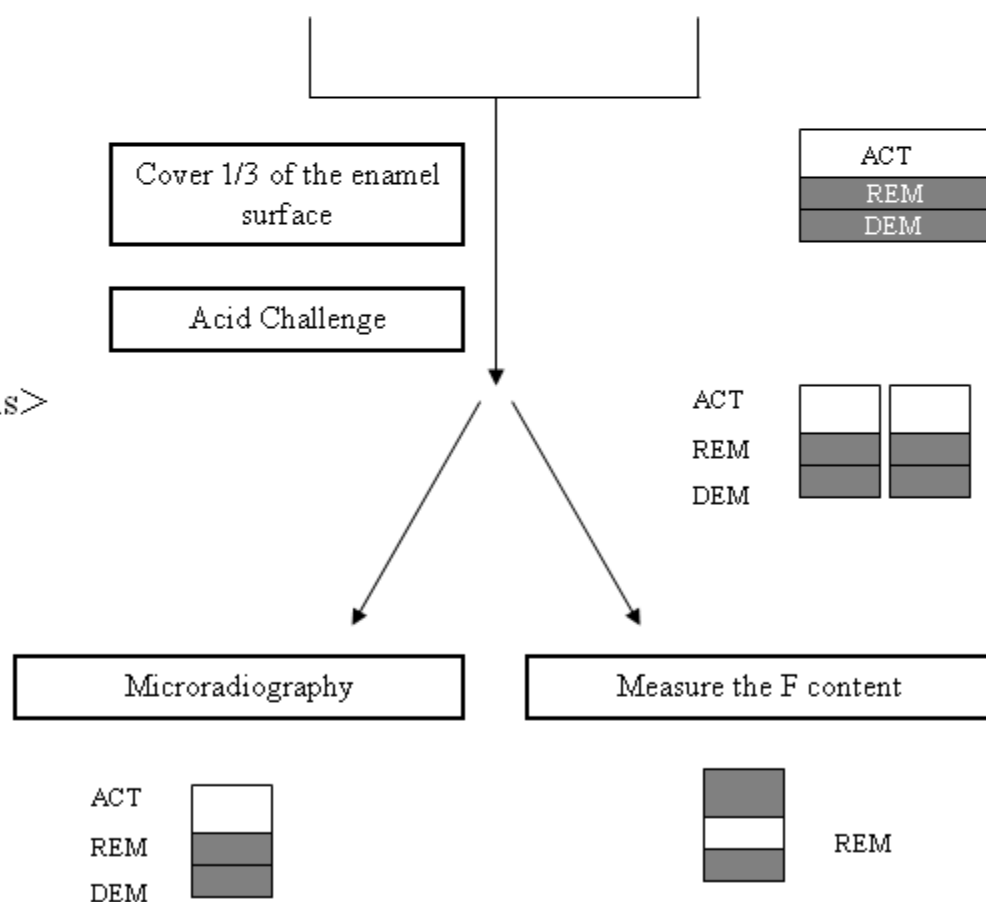


Figure 2: Outline of the experimental procedure

The patients were randomized to a double-blind cross-over protocol with a 1-week washout period between treatments.

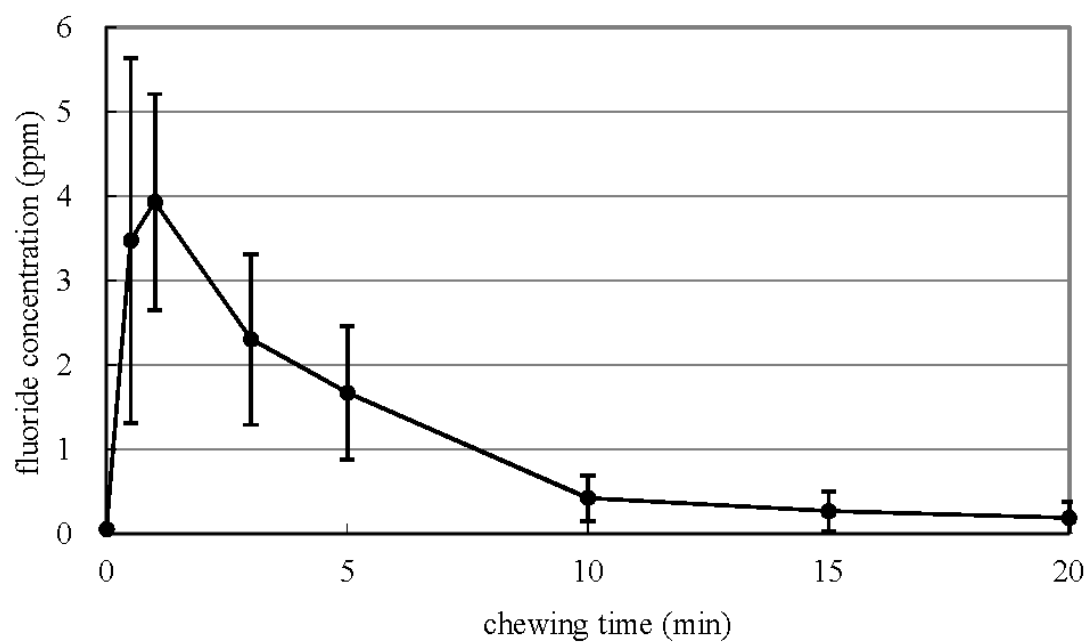


Figure 3: Fluoride concentration (ppm) in saliva after chewing fluoride gum. The vertical bars represent 1 SD.

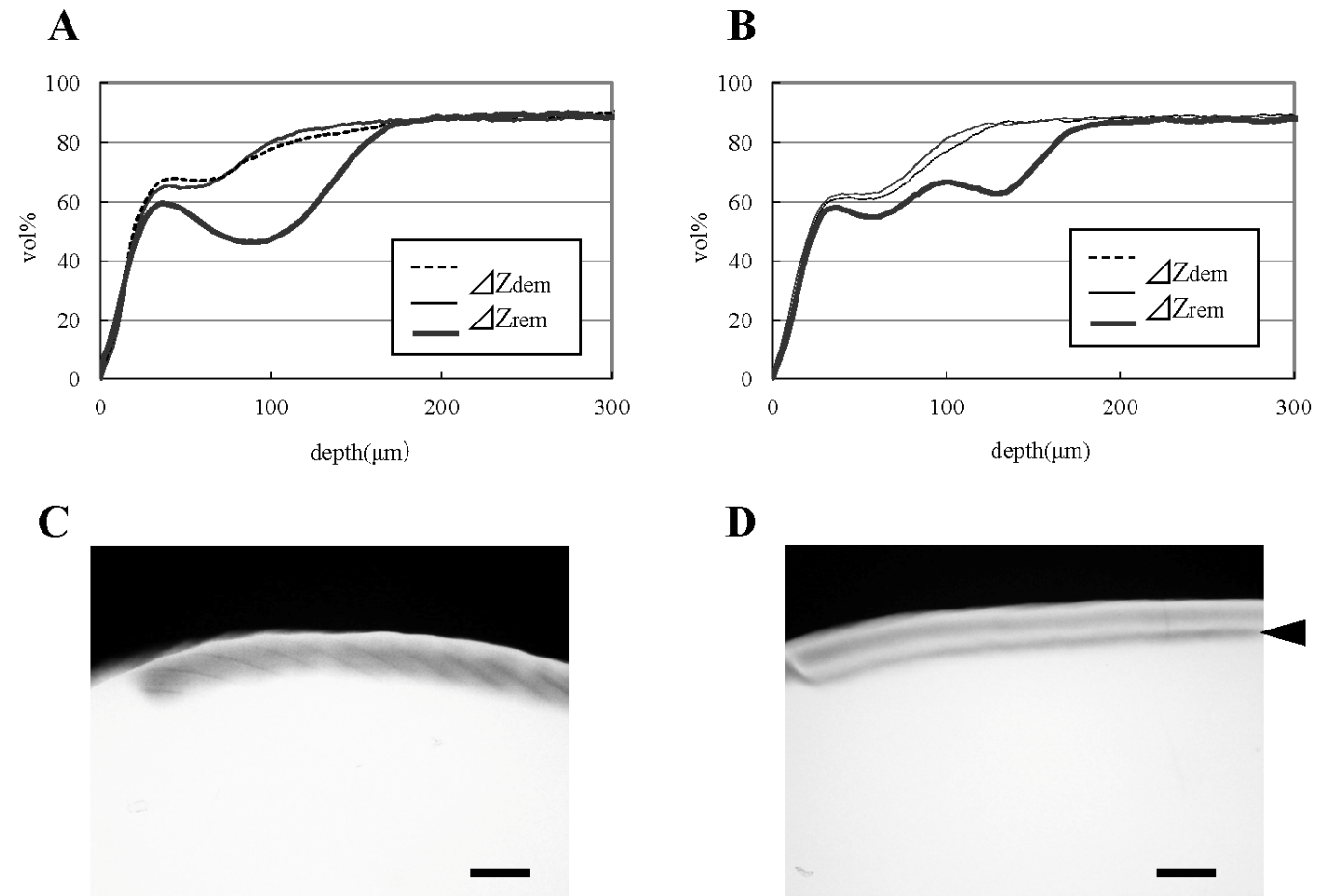


Figure 4: Typical mineral profile of the placebo (A) and FCG (B) groups and microradiographs of the placebo (C) and FCG (D) groups. The laminar structure is indicated by the \blacktriangleleft . Bar = 200 μm

Table 1: Compositions of the sugar-free chewing gums (pellet type)

	FCG(%)	placebo gum(%)
Xylitol	41	41
Maltitol	33	33
Gum base	17	17
Flavors	3	3
Camellia extract MJ	1	0
Colors	0	1
Others	5	5
total	100	100

Table 2: Values of ΔZ_{REM} and ΔZ_{ACT} (Mean \pm SD)

	FCG group		Placebo group	
ΔZ_{REM}	469	\pm 418	270 \pm 427	p<0.03
ΔZ_{ACT}	1699	\pm 1194	2216 \pm 964	p<0.026

Table 3: Fluoride concentration (ppm) in enamel after chewing fluoride and placebo gum
The fluoride levels with FCG were significantly higher than with placebo gum at both depths,
i.e., the surface 0~30 μm and subsurface 30~120 μm ($p<0.001$).

	Surface layer depth (μm)	F concentration (ppm)	t-test
FCG group	35.0 ±3.8	712 ±137	P<0.001
Placebo group	32.9 ±4.1	378 ± 71	
.....			
	Subsurface layer depth (μm)		
FCG group	126.6 ±6.8	656 ±95	P<0.001
Placebo group	126.7 ±6.8	159 ± 26	